Synopsis for Ph.D. work

on

"Identification of small molecule immunomodulators of PD-1 pathway through computational approaches"

by

Lovika Mittal

(Enrolment no. 16/Ph.D./DPSRU/2018)

Under the supervision of:

Dr. Rajiv Kumar Tonk

Associate Professor

Department of Pharmaceutical Chemistry, Delhi Pharmaceutical Sciences and Research University (DPSRU), New Delhi.

Dr. Shailendra Asthana

Scientist C

Drug Discovery Research Centre (DDRC), Translational Health Science and Technology Institute (THSTI), Faridabad.

Annexure-I

Table of Contents

A. Title of the research project

B. Importance of the study

C. Introduction

D. Literature review

E. Hypothesis and specific aims

F. Research framework

G. Expected outcomes

H. References

1. **Title of the research project:** Identification of small molecule immunomodulators of PD-1 pathway through computational approaches.

2. Importance of the study

Our immune system has the ability to distinguish between body's own (normal) cells and the "foreign" cells and attacks on the foreign cells by activating certain molecules known as "*checkpoints*" to trigger an immune response. The immune *checkpoints* can act as stimulatory or inhibitory molecules to maintain equilibrium between T-cells immune responses by either T cells activation or suppression [1].

Cancer or tumor cells either use or express these immune checkpoints to escape the host immune system by suppressing the T-cells[1]. However, the blockade of immune *checkpoints* by exogenously added antagonist activates the immune cells and is effectively being used in the treatment of cancer and infectious diseases[2]. The immune checkpoint therapy has become a "backbone" of immunotherapy[3]. PD-1 and PD-L1 are one of such immune *checkpoints* whose binding leads to T-cells suppression. They are the most validated and key targets for the cancer treatment [4–6]. .

Identification of monoclonal antibodies appears as a major breakthrough in the cancer immunotherapy. FDA approved some monoclonal antibodies (mAbs) which target the PD-1/PD-L1 axis for cancer treatment[7]. Clinical data have shown that antibody blockade of immune *checkpoints* can indeed significantly enhance the anti-tumor activity [8]. However, it has certain drawbacks such as production cost, stability, immunogenicity and oral bioavailability [9,10]. Recently, a nanobody (named as KN035) targeting PD-L1 with high affinity, smaller size, favorable physicochemical properties has been designed and has showed a strong antitumor activity than existing antibodies against it [11]. Furthermore, there are few patented small molecules and peptides that are capable to block the PD-1/PD-L1 interaction and restores the T cell activity [12–14]. However, the development of small molecule inhibitors still lags behind that could be of great profit to mankind in terms of low cost, high stability and oral bioavailability. Therefore, alternative lower molecular weight (LMW) immune checkpoint antagonists are also needed to be investigated at atomic level.

3. **Keywords**: PD-1, PD-L1, protein-protein interactions (PPIs), antagonists, immunomodulators, virtual screening, molecular dynamics.

4. Introduction

4.1 Programmed Cell Death protein (PD-1) and its Ligands (PD-L1 and PD-L2): PD-1 protein in humans is encoded by the *PDCD1* gene[6]. PD-1 is a type I transmembrane protein belonging to CD28 family of receptors. It is expressed on the T cells, B cells, dendritic cells (DCs), NK cells, NK T cells, monocytes and macrophages[2,6]. PD-1 binds two ligands, PD-L1 and PD-L2. These ligands are the cell surface proteins expressed on the Antigen Presenting Cells (APCs). The expression of PD-L1 is much more diverse than PD-L2 as PD-L1 is expressed on both immune cells and non-hematopoietic tissues whereas PD-L2 is expressed only in few cells and tissues[2,6]. The PD pathway has unveiled that binding of PD-1 with PD-L1 or PD-L2 induces T-cell suppression. PD-1 acts like a switch to either suppress the T-cells (T-cell 'off') to avoid attacking body's own cells by binding to PD-L1, or activate T-cells (T-cell 'on') to kill the cancer cells or infectious cells by not binding to PD-L1 (Figure 1). Removal of either of the proteins can result in the breakdown of tolerance and development of autoimmunity[6]. These immune *checkpoints* are active in pregnancy, tissue allografts, and various cancer types. Interestingly, PD-L1 is one of the potential biomarkers being assessed for cancer treatment [2]. PD-L1 is known to be highly upregulated on many kinds of tumor cells, including melanoma, ovarian, and lung cancers thus helping them to escape the host immune system [2]. The PD-1/PD-L1 pathway plays important regulatory role in a various chronic

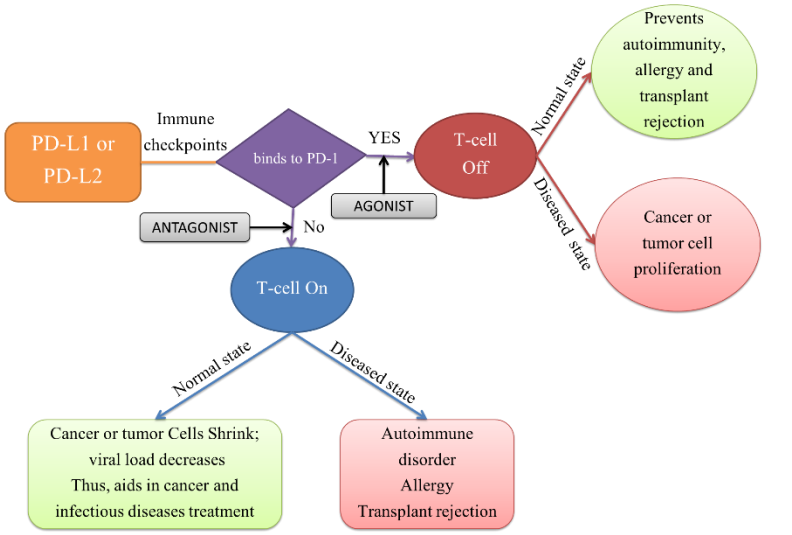

Figure 1: mechanism of inhibition and activation in diseased and normal states highlighting the importance of PD-1/PD-L1 interaction

infections, autoimmune disorders and cancer, providing a platform for developing "a new generation of therapeutic therapies" [2,15].

4.2 Recent knowledge on the immune checkpoint inhibitors: PD-1 and its ligands are the validated and attractive targets for the development of various immunotherapies[4,5]. The immune *checkpoint* inhibitors are emerging as a front-line treatment for several types of cancers by blocking the interaction between PD-1 and PD-L1, thereby, activating the immune response of T-cells and eliminate cancer cells [16–18]. There are FDA approved monoclonal antibodies (mAbs) targeting PD-1/PD-L1 pathway for the range of advanced and metastatic cancers and will be getting approved more[7] (Table 1). These FDA approved antibodies against PD-1 or PD-L1 are being tested in the combination therapies with other drugs for cancer treatment[7]. However, there are some drawbacks with mAbs such as expensive therapy, poor tissue or tumor penetrance, production cost, no oral bioavailability, lesser stability and immunogenicity[9,10]. Therefore to overcome these problems small molecule or low molecular weight (LMW) protein drugs are urgently needed and combination therapy involving antibodies and small molecule inhibitors may become a boon for the treatment of cancers[3,19,20]. There are few nanobodies, peptides and small synthetic inhibitors reported against PD-L1 that showed good results in restoring the T-cell activity for treating cancers[12–14] (Table 1). Nevertheless, it is a challenging task to successfully develop small molecules or peptides as PD-1/PD-L1 involves protein-protein interaction (PPI) whose interface lacks binding cavities to accommodate small molecules[14,21] . It has been observed in clinical trials that antibodies targeting PD-L1 produce lesser immune toxicity and selectively regulates the immune response in the tumor microenvironment [2,22]. Therefore, with all the points into consideration we wish to identify small molecules against PD-1/PD-L1 axis to block their interaction.

Table 1: List of PD-1/PD-L1 antagonists in different stages of development.

Target	Immunotherapeutic Agents	Stage of Development	Type of antagonists
PD-1	Nivolumab (BMS-936558)	FDA approved (2014)	monoclonal antibody
	Pembrolizumab (MK-3475)	FDA approved (2014)	monoclonal antibody

	Cemiplimab	FDA approved (2018)	Monoclonal antibody
	MEDI0680 (AMP-514)	Phase I	monoclonal antibody
	Pidilizumab (CT-011)	Phase II	monoclonal antibody
	Camrelizumab (SHR-1210)	Phase III	monoclonal antibody
	Spartalizumab (PDR001)	Phase II	monoclonal antibody
PD-L1	Atezolizumab (MPDL-3280A)	FDA approved (2016)	monoclonal antibody
	Avelumab (MSB0010718C)	FDA approved (2017)	monoclonal antibody
	Durvalumab(MEDI4736)	FDA approved (2017)	monoclonal antibody
	BMS-936559 (MDX-1105)	Phase II	monoclonal antibody
	Macrocyclic inhibitors	Patented	Peptide
	BMS-202	Patented	small molecule
	AUNP-12 (Aurigene-012)	Patented	Peptide
	CA-170	Phase I	Small molecule

4.3. Challenges in designing small molecule inhibitors against PD-1 or PD-L1: Targeting protein-protein interaction interface is very challenging due to the large, flat, flexible and highly hydrophobic surface area of the PD-1 and PD-L1 interaction interfaces[14,21]. It is quite challenging to design a small molecule that could block the full interface of PD-L1 as monoclonal Antibodies (mAbs) could. Therefore, the identification of the "*hot-spots*" from the key interactions on the interface is very important for the designing of small molecule antagonists.

4.4 Importance and strength of protein structural bioinformatics and computational biophysics: The ability to predict whether a particular protein can bind with high affinity and specificity to small drug like compounds is based solely on its three-dimensional structure. The

structure of the target proteins is the blueprint to understand the structural-dynamical-functional properties. Integration of computational simulations and predictions with experimental validation allows much broader and effective exploration of the structure, function and modulation of the therapeutic targets at atomic level. The crystallographic studies have provided structural insights of the interactive interfaces between PD-1 and its ligands, though the dynamical perspective is barely seen by it[23]. Identification of hotspot residues, structural dynamics and free energy calculations will aid in designing the small molecules targeting this pathway[21]. The crystal structures of human PD-L1 (hPD-L1) complexed with human PD-1 (hPD-1) have indicated that hPD-L1 ectodomain interacts through their CC'FG strands in the IgV domains [24]. It is observed that this same interface is targeted by mAbs, peptides, and small molecules. As PD-L1 belongs to B-7 receptor family which is known to exist in dimers, it is also observed from the crystal structures, that PD-L1 also exists as a dimer in free state as well[15]. From the crystallographic study it is observed that binding of hPD-L1 to hPD-1 induces certain conformational changes in hPD-1 loops[25] (Figure 2). The monoclonal antibodies such as Pembrolizumab and Nivolumab have also caused conformational changes in the hPD-1 loops that were not observed in the monomer state of hPD-1[26]. Binding of small molecules to hPD-L1 induces dimerization of hPD-L1 and significant rearrangements in the side chains at its binding surface areas[27].

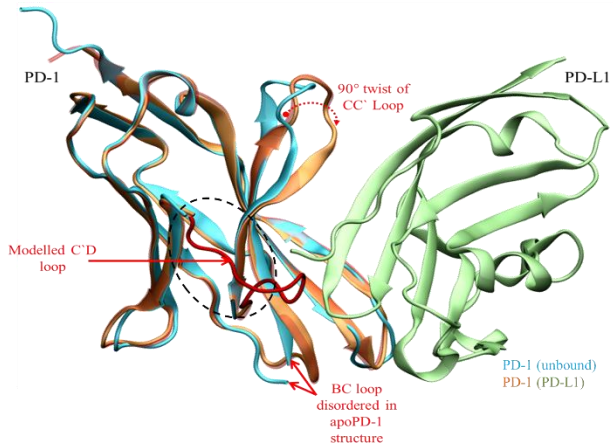

Figure 2: Superimposition of PD-1 monomer on PD-1/PD-L1 dimer

5. Hypothesis and Specific Aims:

Since, the antibody-based immunotherapies come with certain drawbacks including side-effects, the field encourages for the development of small molecules that could overcome the drawbacks of antibodies. Moreover, the molecular basis of anti-PD-1 or anti-PD-L1 activity of reported inhibitors is clear. The crystallographic studies have provided useful insights of the interactive interface of PD-1/PD-L1 but their characterization at the residue wise dynamics

level is still needed to be scrutinized. Therefore, with all points into consideration we would like to apply structure-based drug design approaches for the identification of small molecules. The major aims to be accomplished in this research work are as follows:

a) To explore the structure-dynamics-functional activity of PD-1/PD-L1 in different states such as APO and/or in bound form with their binding partners (COM) and with their known antagonists by using the all atom molecular dynamics simulations.

b) To map the dynamic interface from different partners of PD-1/PD-L1 for the identification of *hot-spot* zones for rational designing of small molecules.

c) To screen a large database of chemical compounds through virtual screening approach against PD-1/PD-L1 interactive interfaces and evaluate their stability and thermodynamic parameters to come up with more diverse set of small inhibitors that could potentially be most likely binders at either PD-L1 or PD-1 to block PD-1/PD-L1 interaction interface.

d) To validate the computationally predicted most likely active molecules "*hits*" in an iterative manner with in-vitro assays.

6. Research framework

Objective 1: To study the PD-1 pathway in normal and diseased conditions.

a) To study the literature for understanding of the PD-1 pathway.
b) To explore PD-1 and its associated interactors (PPIs) in terms of structure, composition, and their druggability.

Objective 2: To curate the repository for PD-1/PD-L1 at its sequence, structural and ligand level.

a) To collect the available structures of PD-1/PD-L1 in bound (monoclonal antibodies (mAbs), peptides, small molecules, or other binding partners) and unbound forms.
b) To annotate the sequence similarities between the proteins.
c) To extract the known information for all reported inhibitors from available resources.

d) To map all the possible interactions between PD-1/PD-L1 with their inhibitors in bound forms.

Objective 3: To explore the molecular basis of recognition of inhibitors to PD-1/PD-L1 (for understanding of their anti-PD-1/anti-PD-L1 activity) through in-silico approaches.

a) To understand the static structures of PPI interface(s) of PD-1/PD-L1 in bound and unbound forms.
b) To understand the structural and conformational changes of PD-1 and PD-L1 upon binding to each other.
c) To understand the binding mode of the inhibitors on the PD-1/PD-L1 and the conformational changes induced in the proteins.
d) To characterize their interactive interface on the basis of structural, dynamics and thermodynamics level.
e) To identify the *hot-spot* zones on both partners for rational designing of small molecules.
f) To understand the possible mechanism of inhibition through comparative analysis between PD-1/PD-L1 intermolecular interfaces and the interfaces shared by their inhibitors.

Objective 4: To identify the small molecules targeting the PD-1/PD-L1 axis by in-silico approaches and their experimental validation.

a) To perform the virtual screening of chemical compounds databases targeting the PD-1/PD-L1 interfaces.
b) To identify the computationally active *hits* by estimation of their stability and binding free energy quantification.
c) To validate the computationally active *hits* by experimental assays.

7. Methodology and techniques to be used during the study.

The study initially will involve Retrieval of three-dimensional structures from Protein Data Bank (PDB) database

I. Computational Approaches:

a) Molecular Dynamics (MD) Simulation
b) Free energy calculations
c) Molecular Docking
d) Interaction fingerprinting
e) *in-silico* prediction of ADME properties of molecules
f) Virtual screening of chemical compounds library

II. Experimental validations of computationally identified hits through in-vitro assays

8. Expected outcomes:

We are applying the structure-based chemo-informatics and computational-biophysical approaches to (a) understand the mechanism of protein-protein interaction (PPI) involved in hPD-1/PD-L1 association and (b) to define structure-activity relationships between the reported compounds and the hPD-1/hPD-L1 with the goal of (c) rational identification of potent small molecules that interact selectively with hPD-1/hPD-L1 interface.

9. References

1. Pardoll, D. M. The blockade of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer* **12**, 252–264 (2012).
2. Chinai, J. M. *et al.* New immunotherapies targeting the PD-1 pathway. *Trends in Pharmacological Sciences* **36**, 587–595 (2015).
3. Huck, B. R., Kötzner, L. & Urbahns, K. Small Molecules Drive Big Improvements in Immuno-Oncology Therapies. *Angew. Chem. Int. Ed Engl.* **57**, 4412–4428 (2018).
4. Iwai, Y. *et al.* Involvement of PD-L1 on tumor cells in the escape from host immune

system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 12293–12297 (2002).

5. Nishimura, H., Nose, M., Hiai, H., Minato, N. & Honjo, T. Development of Lupus-like Autoimmune Diseases by Disruption of the PD-1 Gene Encoding an ITIM Motif-Carrying Immunoreceptor. *Immunity* **11**, 141–151 (1999).
6. Okazaki, T. & Honjo, T. PD-1 and PD-1 ligands: from discovery to clinical application. *Int. Immunol.* **19**, 813–824 (2007).
7. Tang, J. *et al.* Trial watch: The clinical trial landscape for PD1/PDL1 immune checkpoint inhibitors. *Nat. Rev. Drug Discov.* **17**, 854–855 (2018).
8. Alsaab, H. O. *et al.* PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome. *Front. Pharmacol.* **8**, 561 (2017).
9. Chames, P., Van Regenmortel, M., Weiss, E. & Baty, D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br. J. Pharmacol.* **157**, 220 (2009).
10. Kinch, M. S. An overview of FDA-approved biologics medicines. *Drug Discov. Today* **20**, 393–398 (2015).
11. Zhang, F. *et al.* Structural basis of a novel PD-L1 nanobody for immune checkpoint blockade. *Cell Discov* **3**, 17004 (2017).
12. Magiera-Mularz, K. *et al.* Bioactive Macrocyclic Inhibitors of the PD-1/PD-L1 Immune Checkpoint. *Angew. Chem. Int. Ed Engl.* **56**, 13732–13735 (2017).
13. Skalniak, L. *et al.* Small-molecule inhibitors of PD-1/PD-L1 immune checkpoint alleviate the PD-L1-induced exhaustion of T-cells. *Oncotarget* **8**, (2017).
14. Zarganes-Tzitzikas, T. *et al.* Inhibitors of programmed cell death 1 (PD-1): a patent

review (2010-2015). *Expert Opin. Ther. Pat.* **26**, 973–977 (2016).

15. Chen, Y. *et al.* A dimeric structure of PD-L1: functional units or evolutionary relics? *Protein Cell* **1**, 153–160 (2010).
16. Pacheco, J. M., Camidge, D. R., Doebele, R. C. & Schenk, E. A Changing of the Guard: Immune Checkpoint Inhibitors With and Without Chemotherapy as First Line Treatment for Metastatic Non-small Cell Lung Cancer. *Front. Oncol.* **9**, 195 (2019).
17. Jamy, O. & Sonpavde, G. Emerging first line treatment options for bladder cancer: a review of phase II and III therapies in the pipeline. *Expert Opin. Emerg. Drugs* **22**, 347–355 (2017).
18. Mehmi, I. & Hill, J. Ipilimumab with anti PD-1 (nivovlumab or pembrolizumab) after progression on first line anti-PD-1 therapy for advanced melanoma. *Journal of Clinical Oncology* **36**, e21552–e21552 (2018).
19. Konstantinidou, M., Zarganes-Tzitzikas, T., Magiera-Mularz, K., Holak, T. A. & Dömling, A. Immune Checkpoint PD-1/PD-L1: Is There Life Beyond Antibodies? *Angew. Chem. Int. Ed Engl.* **57**, 4840–4848 (2018).
20. Chen, T. *et al.* Peptide-based and small synthetic molecule inhibitors on PD-1/PD-L1 pathway: A new choice for immunotherapy? *Eur. J. Med. Chem.* **161**, 378–398 (2019).
21. Kuenemann, M. A. *et al.* In silico design of low molecular weight protein-protein interaction inhibitors: Overall concept and recent advances. *Prog. Biophys. Mol. Biol.* **119**, 20–32 (2015).
22. Kim, J. M. & Chen, D. S. Immune escape to PD-L1/PD-1 blockade: seven steps to success (or failure). *Ann. Oncol.* **27**, 1492–1504 (2016).
23. Zak, K. M. *et al.* Structural Biology of the Immune Checkpoint Receptor PD-1 and Its

Ligands PD-L1/PD-L2. *Structure* **25**, 1163–1174 (2017).

24. Lin, D. Y.-W. *et al.* The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors. *Proceedings of the National Academy of Sciences* **105**, 3011–3016 (2008).
25. Zak, K. M. *et al.* Structure of the Complex of Human Programmed Death 1, PD-1, and Its Ligand PD-L1. *Structure* **23**, 2341–2348 (2015).
26. Lee, J. Y. *et al.* Structural basis of checkpoint blockade by monoclonal antibodies in cancer immunotherapy. *Nat. Commun.* **7**, 13354 (2016).
27. Guzik, K. *et al.* Small-Molecule Inhibitors of the Programmed Cell Death-1/Programmed Death-Ligand 1 (PD-1/PD-L1) Interaction via Transiently Induced Protein States and Dimerization of PD-L1. *J. Med. Chem.* **60**, 5857–5867 (2017).